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TITLE

**ASSAYS AND PEPTIDE SUBSTRATE FOR DETERMINING AGGRECAN
DEGRADING METALLO PROTEASE ACTIVITY**

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CROSS REFERENCE TO EARLIER FILED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/053850 filed on July 25, 1997 and U.S. Provisional Application No. 60/055836 filed on August 15, 1997 and U.S. Provisional Application No. 60/062,169 (unknown at filing), filed on October 16, 1997.

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FIELD OF THE INVENTION

This invention is directed to various assays for determining aggrecanase or aggrecan degrading metallo protease (ADMP) activity. This invention also relates to a peptide that acts as a substrate for ADMPs, its use in various assays to determine the presence or absence of (ADMP) activity, and its use as an inhibitor of ADMP activity.

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BACKGROUND OF THE INVENTION

Aggrecan is the major proteoglycan of cartilage and provides this tissue with its mechanical properties of compressibility and elasticity. In arthritic conditions one of the earliest changes observed in cartilage morphology is the depletion of aggrecan [Mankin et al. (1970) J. Bone Joint Surg. 52A, 424-434], which appears to be due to an increased rate of degradation.

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The aggrecan molecule is composed of two N-terminal globular domains, G1 and G2, which are separated by an approximately 150 residue interglobular domain (IGD), followed by a long central glycosaminoglycan (GAG) attachment region and a C-terminal globular domain, G3

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5 [Hardingham et al. (1992) in Articular Cartilage and
Osteoarthritis: Aggrecan, The Chondroitin Sulfate/Keratan
Sulfate Proteoglycan from Cartilage (Kuettnner et al.) pp. 5-
20, Raven Press, New York and Paulson et al. (1987) Biochem.
J. 245, 763-772]. These aggrecan molecules interact through
10 the G1 domain with hyaluronic acid and a link protein to
form large molecular weight aggregates which are trapped
within the cartilage matrix [Hardingham et al. (1972)
Biochim. Biophys. Acta 279, 401-405, Heinegard et al.
(1974) J. Biol. Chem. 249, 4250-4256, and Hardingham, T.E.
15 (1979) Biochem. J. 177, 237-247]. Loss of aggrecan from
cartilage in arthritic conditions involves proteolytic
cleavage of the aggrecan core protein within the IGD,
producing a N-terminal G-1 fragment that remains bound to
hyaluronic acid and the link protein within the matrix,
20 releasing a large C-terminal GAG-containing aggrecan
fragment that diffuses out of the cartilage matrix. Loss of
the C-terminal fragment results in cartilage deficient in
its mechanical properties. This deficiency arises because
the GAGs which are present on the C-terminal portion of the
25 aggrecan core protein are the components of aggrecan that
impart the mechanical properties to the molecule through
their high negative charge and water binding capacity.

Two major sites of proteolytic cleavage have been
identified within the IGD, one between amino acid residues
30 Asn³⁴¹-Phe³⁴² and the other between amino acid residues
Glu³⁷³-Ala³⁷⁴ (human sequence enumeration). Although G1
fragments formed by cleavage at the Asn³⁴¹-Phe³⁴² site and at
the Glu³⁷³-Ala³⁷⁴ site have been identified within articular
cartilage [Flannery et al. (1992) J. Biol. Chem. 267, 1008-
35 1014], the only N-terminus identified on GAG-containing
aggrecan C-terminal aggrecan fragments in synovial fluids of
patients with osteoarthritis [Sandy et al. (1992) J. Clin.
Invest. 69, 1512-1516], inflammatory joint disease
[Lohmander et al. (1993) Arthritis Rheum. 36, 1214-1222] and

5 in the media from cartilage explant and chondrocyte cultures
stimulated with interleukin-1 or retinoic acid [Sandy et al.
(1991) J. Biol. Chem. 266, 8198., Sandy et al. (1991) J.
Biol. Chem. 266, 8683-8685., Leulakis et al. (1992) Biochem.
J. 264, 589-593., Ilic et al. (1992) Arch. Biochem.
10 Biophys. 294, 115-122., Lark et al. (1995) J. Biol. Chem.
270, 2550-2556.] is ARGSVIL, indicating that they were
formed by cleavage between amino acid residues Glu³⁷³-Ala³⁷⁴.
These observations suggest that cleavage at this site may be
responsible for cartilage degradation.

15 Although many matrix metalloproteases (MMP-1, -2, -
3, -7, -8, -9 and 13) have been shown to cleave in vitro
at the Asn³⁴¹-Phe³⁴² site, digestion of aggrecan with a
number of these purified proteases has not resulted in
cleavage at the Glu³⁷³-Ala³⁷⁴ site [Fosang et al. (1992)
20 J. Biol. Chem. 267, 19470-19474., Flannery et al. (1992)
J. Biol. Chem. 267, 1008-1014., Fosang et al. (1993)
Biochem. J. 295, 273-276., Fosang et al. (1996) FEBS
Lett. 380, 17-20., Flannery et al. (1993) Orthop. Trans.
17, 677., and Fosang et al. (1994) Biochem. J. 305, 347-
25 351]. Therefore, cleavage at this site has been
attributed to a novel, proteolytic activity,
"aggrecanase".

In addition to the Glu³⁷³-Ala³⁷⁴ bond within the
interglobular domain of aggrecan, four potential
30 aggrecanase-sensitive sites have been identified within
the C-terminus of the aggrecan core protein [Loulakis et
al. (1992) Biochem. J. 264, 589-593. and Sandy et al.
(1995) Acta Orthop Scand (Suppl 266) 66, 26-32].
Although cleavage at these sites which are not within the
35 interglobular domain would not be expected to release the
major portion of the aggrecan molecule from the matrix,
they may be involved in earlier processing of aggrecan
within the matrix.

5 It follows from the foregoing considerations that a
sensitive and specific assay that can detect such
aggrecanase activity of aggrecan degrading metallo
proteases (ADMPs) would be beneficial in helping to
identify inhibitors of members of the ADMP family, which
10 could serve as potential therapeutic agents for treating
aggrecanase-related disorders cited above.

SUMMARY OF THE INVENTION

15 A preferred embodiment of the invention provides
assays that determine the presence of aggrecan degrading
metallo protease (ADMP) activity.

20 A preferred embodiment of the invention provides an
assay using purified native aggrecan or recombinant
aggrecan as the substrate and monitoring product
generation via a direct enzyme-linked immunosorbent assay
(ELISA) using neoepitope antibodies to detect the new N-
terminus or new C-terminus on aggrecan fragments formed
25 by specific cleavage at an ADMP-sensitive site in the
aggrecan core protein.

A preferred embodiment of the invention provides
peptides that have been found to act as substrates for
the family of aggrecan degrading metallo proteases
30 (ADMPs). One peptide, based on the human aggrecan
sequence around the Ala373-Glu374 ADMP-sensitive site has
the sequence:

QTVTWPDMELPLPRNITEGE-ARGSVILTVKPIFEVSPSPL

(SEQ ID No:1)

35 A second peptide, based on the bovine aggrecan sequence
around the Ala373-Glu374 ADMP-sensitive site has the
sequence:

QTVTWPDVLELPLPRNITEGE-ARGSVILTAKPDFEVSP~~TAPE~~

(SEQ ID NO:2)

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5 Both peptides are capable of being cleaved at this
specific recognition site by members of the family of
ADMP proteins. A third peptide, based on the human
aggrecan sequence around the Ala1714-Gly1715 ADMP-
sensitive site has the sequence:

10 ITFVDTSLVEVTPPTTFKEEE-GLGSVELSGLPSGELGVSGT

(SEQ ID NO:3)

and is capable of being cleaved at this specific
recognition site by the family of ADMP proteins.

A preferred embodiment of the invention provides
15 assay formats and methods of utilizing these peptide
substrates for the detection and quantification of ADMP
activity.

A preferred embodiment of the invention provides a
modified version of the peptide substrates and a method
20 for their use as an inhibitor of ADMP activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention have been
25 chosen for purposes of illustration and description, but
are not intended in any way to restrict the scope of the
invention. The preferred embodiments of certain aspects
of the invention are shown in the accompanying drawings
described below.

30 Figure 1. Shows the activity of a biotinylated form of
the 41-mer peptide substrate (41-PS) against ADMP
enzymatic activity using the microplate assay format and
the inhibition of that activity by a hydroxymate
35 inhibitor compound.

Figure 2. Shows the activity of a biotinylated form of
the 41-mer peptide substrate (41-PS) against ADMP
enzymatic activity using the HPLC assay format.

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Figure 3. Shows the activity of the biotinylated form of the 41-mer peptide substrate (41-PS) against ADMP enzymatic activity using the microplate assay format and the inhibition of that activity by the 30-mer inhibitor peptide (30-IP), QTVTWPDMEPLPRNITEGQARGSVILTVK-Biotin, the sequence of which is based upon the sequence of the 41-PS.

DETAILED DESCRIPTION OF THE INVENTION

15 A family of aggrecan degrading metallo protease (ADMP) proteins cleave the aggrecan core protein at the Glu³⁷³-Ala³⁷⁴ peptide bond and thus exhibit the enzymatic activity referred to as "aggrecanase" activity [Flannery et al. (1992) J. Biol. Chem. 267, 1008-1014]. The
20 presence of ADMP enzymatic activity can be determined by monitoring the production of aggrecan fragments generated exclusively by cleavage at the Glu³⁷³-Ala³⁷⁴ peptide bond within the aggrecan core protein. These aggrecan fragments are detected by using neoepitope antibodies to
25 the new N-terminus or new C-terminus on fragments produced by specific cleavage at this ADMP-sensitive site. The neoepitope antibodies used encompass but are not limited to, the BC-3 monoclonal antibody (Hughes, C.E., et al., Biochem. J. 306:799-804, 1995) as first
30 described in U.S. Provisional Patent Application Serial Number 60/006,684 and subsequently described in U.S. Patent Application Serial Number 08/743,439.

ADMP activity may also be detected by monitoring the production of fragments formed by cleavage at alternative
35 ADMP-sensitive sites using neoepitope antibodies to the new C-terminus or to the new N-terminus generated by ADMP-specific cleavage at these sites. Alternative sites in the aggrecan core protein encompass, but are not limited to, the E1545-G1546, E1714-G1715, E1819-A1820, or

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5 E1919-L1920 bond (numbering based on the human aggrecan
core protein sequence.

A preferred assay format involves using purified
native aggrecan or recombinant aggrecan as the substrate
with product detection via a direct enzyme-linked
10 immunosorbent assay (ELISA), herein referred to as the
"Problot assay", using neoepitope antibodies to the new
C-terminus or new N-terminus on aggrecan fragments
generated upon specific cleavage at ADMP-sensitive sites
within the aggrecan core protein. Alternative sites in
15 the aggrecan core protein encompass, but are not limited
to, the E1545-G1546, E1714-G1715, E1819-A1820, or E1919-L1920
bond (numbering based on the human aggrecan core protein
sequence). These human aggrecan ADMP-sensitive cleavage
sites are conserved in aggrecan from various animal
20 species although the absolute numbering based on the
sequence of the aggrecan core protein may vary from
species to species. Conserved amino acid sequences in
various species around conserved ADMP-sensitive sites are
shown below.

25	Human	NITEGE ³⁷³	³⁷⁴ ARGSVILT
	Bovine	NITEGE	ARGSVILT
	Rat	NITEGE	ARGNVILT
	Mouse	NVTEGE	ALGSVILT
30	Pig	NITEGE	ARGTVILT
	Sheep	NITEGE	ARGNVILT
	Chicken	NVTEEE	ARGSI
	Horse	NITEGE	ARGNVILT
35	Human	ASTASELE ¹⁵⁴⁵	¹⁵⁴⁶ GRGTIGIS
	Bovine	ATTAGELE	GRGTIDIS
	Mouse	ATTSSELE	GRGTIGIS
	Rat	ATTASELE	GRGTISVS
40	Human	PTTFKEEE ¹⁷¹⁴	¹⁷¹⁵ GLGSVELS
	Bovine	PTTFKEEE	GLGSVELS
	Rat	PTTFREEE	GLGSVELS
	Mouse	PTTFREEE	GLGSVELS

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5 Human TQAPTAQE¹⁸¹⁹ 1820 AGEGPSGI
Bovine TQAPTAQE AGEGPSGI
Rat TLAPTAQE AGEGPSSI
Mouse TQAPTAQE AGEGPSGI
10 Chicken TQTSVAQE VGEGPSGM

Human TEPTISQE¹⁹¹⁹ 1920 LGQRPPVT
Bovine TEPTVSQE LGQRPPVT
Rat TEPTVSQE LGHGPSMT
15 Mouse TEPTVSQE LGHGPSMT
Chicken TRPTVSQE LGGETAVT
Dog TEPTVSQE LAQRPPVT

20 Thus, aggrecan from various animal species,
including but not limited to, bovine, dog, pig, rat,
mouse, sheep, horse and chicken may also be used as a
substrate for detecting ADMP activity.

The direct ELISA assay employs 96-well filtration
25 plates containing polyvinyl-denedifluoride (PVDF)
cationically charged membranes. These membranes are
semi-selective in binding the highly negatively-charged
aggrecan, which allows for binding of detectable levels
of neoepitope antibody-reactive aggrecan fragments from
30 solutions containing high levels of other proteins.

Utilizing neoepitope antibodies allows detection of
fragments formed specifically by ADMP-mediated cleavage
even in the presence of other proteolytic activities
which may be present in crude preparations. Thus, the
35 Problot assay can be used to monitor ADMP activity in
culture medium containing other proteases, as well as to
monitor the activity of the purified ADMP enzyme.
Therefore, this assay has particular use in following
ADMP activity during purification from tissue or media
40 samples as well as for use in enzymatic assays to
evaluate inhibitors of the ADMP enzyme. The Problot
assay can also be used to detect ADMP-generated aggrecan
fragments in culture media from tissue or cell cultures
stimulated to induce ADMP-mediated degradation. This

5 assay may also be useful for detecting ADMP-generated
aggrecan fragments in cartilage, synovial fluid, serum,
urine or other biological samples from patients with
ADMP-associated diseases.

10 Peptide substrates are commonly employed in a
variety of assays to determine the presence of enzymes
that catalyze the hydrolysis of proteins. One skilled in
the art would rely on the use of peptide substrates that
are relatively short in length, generally consisting of
approximately six to ten amino acids in length. These
15 peptide substrates typically encompass amino acid
sequences that bracket the known hydrolysis site of the
natural protein substrates. These peptide substrates,
including those for matrix metalloproteases, serine
proteases, aspartyl proteases, and aminopeptidases, are
20 readily available for use in a variety of enzymatic
assays.

This invention provides a peptide that has been
found to act as a substrate for the family of ADMPs. It
is commonly known that short peptide sequences which
25 contain the proper substrate cleavage site are quite
acceptable substrates for many proteases (Copeland, R.A.,
Enzymes: A Practical Introduction to Structure,
Mechanism and Data Analysis, VCH/Wiley, New York, 1996).
However, no such peptide, even those containing as many
30 as twenty amino acids, has been determined that will act
as a suitable substrate for ADMPs. The peptides of the
instant invention are unique in that it was unexpectedly
found that these longer, forty amino acid sequence acted
as very good substrates for ADMPs. One such peptide
35 provided by the invention, of the sequence

QTVTWPDMEPLPRNITEGE-ARGSVILTVKPIFEVSPSPL (SEQ ID NO:1)

5 comprises a 40 amino acid segment of the human aggrecan
protein that contains the ITEGE373-374ARGS cleavage site
present in the natural protein substrate, aggrecan, and
is capable of being cleaved at this specific recognition
site by the ADMPs. Since the human aggrecan ADMP-
10 sensitive cleavage sites are conserved in aggrecan from
various animal species, peptides based on the amino acid
sequence around the ADMP-sensitive cleavage sites from
other species can also serve as substrates for ADMPs. A
peptide substrate, similar to SEQ ID NO:1, based on a 41
15 amino acid segment of the bovine aggrecan protein, of the
sequence

QTVTWPDVLPPLPRNITEGE-ARGSVILTAKPDFEVSPTAPE (SEQ ID NO:2)

20 containing the E373-A374 cleavage site is also capable of
being cleaved at this specific recognition site by the
ADMPs.

Cleavage products are easily detected by using
neoepitope antibodies to the N-terminal or C-terminal
25 fragments produced by specific cleavage at the E373-A374
bond, encompassing, but not limited to, the monoclonal
antibody BC-3 (Hughes, C.E., et al., Biochem. J. 306:799-
804, 1995). The BC-3 antibody recognizes the new N-
terminus, ARGS, which is the amino terminal portion of
30 one of the product peptides resulting from the ADMP
activity of the enzyme.

One skilled in the art could readily design peptides
of similar size encompassing the alternative ADMP-
sensitive cleavage sites in the aggrecan core protein,
35 encompassing, but not limited to, regions of the molecule
containing the E1545-G1546, E1714-G1715, E1819-A1820, or
E1919-L1920 bond (numbering based on the human aggrecan

5 core protein sequence). One such peptide provided by the invention, of the sequence

ITFVDTSLVEVTPPTTFKEEE-GLGSVELSGLPSGELGVSGT (SEQ ID NO:3)

10 comprises a 40 amino acid segment of the human aggrecan protein that contains the KEEE¹⁷¹⁴⁻¹⁷¹⁵GLGS cleavage site present in the natural protein substrate, aggrecan, and is capable of being cleaved at this specific recognition site by the ADMPs.

15 When a preferred form of a peptide substrate, biotinylated at the carboxy terminus or amino terminus, is employed, several streptavidin coated supports may be used. These include, but are not limited to microplates, metallic and non-metallic beads, and membranes.

20 Another preferred assay format involves the direct analysis, by high-performance liquid chromatography (HPLC), of the cleavage fragments from the substrate that are generated by ADMP activity.

Another preferred embodiment of the invention provides that a peptide substrate of this invention may be reversed in its role. With proper modification at the P1 position the substrate may be turned into an inhibitor of ADMP activity. Specifically it was found that esterification of the P1 glutamic acid residue (GLU³⁷³) of the substrate peptide SEQ ID NO:1 or its replacement by glutamine abolish catalytic hydrolysis. Unexpectedly, the peptide containing the GLU to GLN substitution at amino acid position 373 (the P1-glutamine containing peptide) was shown to be a competitive inhibitor of the enzyme. Thus, a carboxylate residue at position P1 of the substrate appears to be critical for turnover by ADMPs, but exerts less influence over initial substrate binding to the enzyme. This feature can be readily

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5 exploited by one trained in the art to design specific
peptide and non-peptide inhibitors of this enzyme.

DEFINITIONS

As used herein, the following terms and expressions
10 have the indicated meanings.

The term "aggrecan degrading metallo protease"
("ADMP") activity as referred to herein, refers to the
enzymatic activity of a family of polypeptides which
specifically cleave the protein aggrecan within the
15 interglobular domain at the Glu³⁷³-Ala³⁷⁴ peptide bond,
but do not readily cleave at the Asn³⁴¹-Phe³⁴² bond which
is preferentially cleaved by matrix metalloproteinases.

The term "amino acid" as used herein means an
organic compound containing both a basic amino group and
20 an acidic carboxyl group.

The term "amino acid residue" as used herein means
that portion of an amino acid (as defined herein) that is
present in a peptide.

The term "peptide" as used herein means a compound
25 that consists of two or more amino acids (as defined
herein) that are linked by means of a peptide bond. The
term "peptide" also includes compounds containing both
peptide and non-peptide components, such as pseudopeptide
or peptide mimetic residues or other non-amino acid
30 components. Such a compound containing both peptide and
non-peptide components may also be referred to as a
"peptide analog".

The term "peptide bond" means a covalent amide
linkage formed by loss of a molecule of water between the
35 carboxyl group of one amino acid and the amino group of a
second amino acid.

The term "substrate" refers to a molecule that is
bound by the active site and acted upon by the enzyme.

5 The term "solid-phase peptide synthesis" refers to the direct chemical synthesis of peptides utilizing an insoluble polymeric support as the anchor for the growing peptide, which is built up one amino acid at a time using a standard set of reactions in a repeating cycle

10 (Merrifield, R.B., Science 232, 341-347 1986).

As used herein, the term "TMB" refers to 3,3',5,5'-tetramethylbenzidine.

15 The term "neoepitope antibody" refers to an antibody which specifically recognizes a new N-terminal amino acid sequence or new C-terminal amino acid sequence generated by proteolytic cleavage but does not recognize these same sequences of amino acids when they are present within the intact protein.

20 As used herein, the cleavage site "E373-374A" refers to the ITEGE373-374ARGS bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in aggrecan from various animal species, the cleavage site "E1545-1546G" refers to the SELE1545-1546GRGT bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in aggrecan from various animal species, the cleavage site "E1714-1715G" refers to the KEEE1714-1715GLGS bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in aggrecan from various animal species, the cleavage site "E1819-1820A" refers to the TAQE1819-1820AGEG bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in aggrecan from various animal species, the cleavage site "E1919-1920L" refers to the ISQE1919-1920LGQR bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in aggrecan from various animal species.

The term "aggrecan" as used herein refers to the aggregating proteoglycan, aggrecan, of cartilage from human or various animal species, as the native aggrecan

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5 isolated from tissue, as recombinant full-length aggrecan
or as a recombinant protein representing a portion of the
aggrecan molecule.

As used herein the term "ADMP-susceptible cleavage
site" refers to the E373-374A bond, the E1545-1546G bond,
10 the E1545-1546G bond, the E1819-1820A bond, and the E1919-
1920L bond of aggrecan from human and various animal
species, and to a peptide bond of a protein containing an
amino acid sequence which has a glutamine in the P1
position and shows at least 65% homology with the P1, P2,
15 P3, P1', P2' and P3' amino acids of one or more of the
ADMP-sensitive sites in the aggrecan molecule.

The term "sissel bond" refers to the peptide bond of
a polypeptide that is to be cleaved by a protease. The
term "P1" as used herein refers to the amino acid residue
20 on the N-terminal side of the sissel bond. The term "P2"
as used herein refers to the amino acid residue adjacent
to P1 on the N-terminal side of the sissel bond. The term
"P3" as used herein refers to the amino acid residue
adjacent to P2 on the N-terminal side of the sissel bond.
25 The term "P1'" as used herein refers to the amino acid
residue on the C-terminal side of the sissel bond. The
term "P2'" as used herein refers to the amino acid
residue adjacent to P1' on the C-terminal side of the
sissel bond. The term "P3'" as used herein refers to the
30 amino acid residue adjacent to P2' on the C-terminal side
of the sissel bond.

The term "BC-3 antibody" refers to a monoclonal
antibody that reacts specifically with the newly-formed
amino-terminal sequence ARGS on fragments produced by
35 proteolytic cleavage at the Glu³⁷³-Ala³⁷⁴ aggrecan
cleavage site, but does not recognize this same sequence
of amino acids when they are present within the intact
interglobular domain of the protein (Hughes, C.E., et
al., Biochem. J. 306:799-804, 1995).

5 The term "SEQ ID NO:1" refers to the peptide
sequence QTVTWPDMEPLPRNITEGE-ARGSVILTVKPIFEVSPSPL. The
term "SEQ ID NO:2" refers to the peptide sequence
QTVTWPDVELPLPRNITEGE-ARGSVILTAKPDFEVSPTAPE. The term "SEQ
ID NO:3" refers to the peptide sequence
10 ITFVDTSLVEVTPTTFKEEE-GLGSVELSGLPSGELGVSGT. The term "41-
PS" and "SEQ ID NO:4" refer to the peptide sequence:
QTVTWPDMEPLPRNITEGEARGSVILTVKPIFEVSPSPL-(BIOTINYL)K.
The term "SEQ ID NO:5" refers to the peptide sequence:
ARGSVILTVKPIFEVSPSPL-(BIOTINYL)K. The term "SEQ ID NO:6"
15 refers to the peptide sequence: K(BIOTINYL)-
QTVTWPDMEPLPRNITEGE. The term "30-IP" and "SEQ ID NO:7"
refer to the peptide sequence
QTVTWPDMEPLPRNITEGQARGSVILTV-(BIOTINYL)K.

The invention can be further understood by the
20 following examples. These examples provide an
illustration of embodiments of the invention and should
not be construed to limit the scope of the invention
which is set forth in the appended claims. In the
following examples all methods described are conventional
25 unless otherwise specified.

Example 1

Microplate Assay Format for Detection of ADMP Activity

30 The substrate and product peptides were prepared in
the following manner. A 41 amino acid form (41-PS) SEQ
ID NO:4 of the peptide substrate SEQ ID NO:1 was prepared
by solid phase peptide synthesis. The peptide was
prepared commercially (Quality Controlled Biochemicals,
35 Inc. Hopkinton, MA) as a biotin conjugate by adding an
additional lysine residue at the carboxy terminus of the
peptide SEQ ID NO:1. Biotin was then covalently attached
through the lysine ε-amino side chain.
41-PS sequence:

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5 QTVTWPDMEPLPRNITEGE-ARGSVILTVKPIFEVSPSPL-(BIOTINYL)K
(SEQ ID NO:4)

A 21 amino acid peptide representing the product of ADMP-mediated cleavage of the 41-PS containing the ARGS N-terminus was prepared in a similar manner and had the
10 following sequence:

ARGSVILTVKPIFEVSPSPL-(BIOTINYL)K
(SEQ ID NO:5)

The substrate and product peptide microplates were prepared in the following manner. A 0.1 mM stock of
15 41-PS was made by dissolving it in distilled water. From this a working solution of 7×10^{-8} M 41-PS was prepared in 1X PBS, 0.05% Tween 20. Aliquots of 100 μ L of this solution were added to the microplate wells of a streptavidin coated microtiter strip plate (DUPONT, NEN
20 Products, Catalog # NEF-711). The solutions were allowed to sit at 25°C overnight in order for the biotin moiety of the peptides to bind to the streptavidin which was coated on the microplate. The plate was washed three times with 200 μ L of 1X PBS, 0.05% Tween 20, after which the plate
25 was inverted, blotted dry, sealed and stored at 4°C.

The assay was performed in the following manner. Microplate strips (eight wells each) were rinsed once with 100 μ L of 1X Assay Buffer (Assay Buffer consists of: 50 mM Tris, pH 7.5, 10 mM CaCl_2 , and 100 mM NaCl)
30 and blotted dry. Reactions were prepared in duplicate in a final volume of 100 μ L, containing: 50 μ L of 2X Assay Buffer (100 mM Tris, pH 7.5, 20 mM CaCl_2 , and 200 mM NaCl), 25 μ L of a hydroxymate inhibitor compound (final concentrations consisting of 5.0, 1.0, 0.75, 0.5, 0.25,
35 0.125, 0.05, 0.001, and 0.0 μ M), and 25 μ L of soluble ADMP (0.05 η M). The microplate strips were incubated for 3 hours at 37°C. The microplate wells were then washed six times with 200 μ L of 1X PBS, 0.05% Tween 20 using a Denley Well Wash 4 micro plate washer.

5 A BC-3 antibody solution was prepared by adding 4 μ L
of BC-3 antibody (0.405 mg/mL in PBS) to 2 mL of antibody
dilution buffer (DB), which consisted of: 0.1 g BSA
(Boehringer Mannheim Catalog 238-031), 10 mL 1X PBS, and
10 μ L Tween 20. 100 μ L of this solution was added to
10 each well. The microplate strips were incubated for 1
hour at 25°C. The microplate wells were washed six times
with 200 μ L of 1X PBS, 0.05% Tween 20.

The secondary (detection) antibody solution was
prepared by adding 4 μ L of Goat anti-Mouse-HRP antibody
15 conjugate (Pierce Cat# 31430) (0.8 mg/mL in PBS) to 2
mL of antibody dilution buffer (DB). 100 μ L of this
solution was added to each well. The microplate strips
were incubated for 1 hour at 25°C. The microplate wells
were washed six times with 200 μ L of 1X PBS, 0.05% Tween
20. TMB Substrate (100 μ L per well, DAKO Cat S1600) was
20 added and the microplate strips incubated at 25°C for 15
minutes. The reaction was quenched with 100 μ L of 1N
HCl. The optical density was read at 450nm using a
Molecular Devices Spectromax 250 microplate reader.
25 By coating the plate with the product peptide, one can
create a standard curve which can be used to convert
optical density values to units of activity.

ADMP activity can easily be followed by this method
and inhibition of ADMP activity can be monitored. The
30 IC₅₀ for the inhibition of ADMP by the hydroxamate
inhibitor tested was 0.413 μ M.

Example 2

HPLC Assay for Detection of ADMP Activity

35

The High Performance Liquid Chromatography (HPLC)
instrument used for the assay was from Hewlett-Packard,
model number HP1090, equipped with a HP ChemStation. A
250 x 4.6 mm Vydac C₁₈ column with 10 μ particle size was

5 obtained from The Separations Group, Tesperia, CA; HEPES
from Research Organics, Cleveland, OH; Brij-35 from
Technicon Corp., Tarrington, NY; Other chemicals from
Sigma, St. Louis, MO; the lyophilized peptide substrate
41-PS, QTVTWPDMEPLRLNITEGEARGSVILTVKPIFEVSPSPL-
10 (BIOTINYL)K (SEQ ID NO:4), which includes the ADMP E373-
A374 cleavage site, was obtained from Quality Controlled
Biochemicals, Inc. Hopkinton, MA. A 21 amino acid
product peptide (SEQ ID NO:5) with the sequence,
ARGSVILTVKPIFEVSPSPL-(BIOTINYL)K, used as a standard for
15 quantitation of product formation, was obtained from the
same vendor.

The HPLC assay is performed in the following manner.
The reaction buffer contains 50 mM HEPES, 10 mM CaCl₂,
100 mM NaCl and 0.05% Brij-35, pH 7.5. 30 μM 41-PS was
20 incubated with 1 unit ADMP activity (1 unit = the amount
of ADMP activity resulting in 1 pMole of 21-mer peptide
product produced per hr at 37°) at 37°C for 2.5 hours and
then the reaction was quenched with 50 mM EDTA. A 10 μL
portion of the reaction mixture was injected onto a
25 reverse-phase HPLC C₁₈ column. The peptides were eluted
with a mobile phase of 0.1% trifluoroacetic acid and a
25-45% acetonitrile gradient in 20 minutes. UV
absorbance was measured at 220nm and peak integration was
performed on a Hewlett-Packard HP ChemStation. The 21-
30 mer product peptide was used as a standard for
quantitation of product formation. The 41-PS and 21-mer
product are well separated with retention times of 14.2
and 10.5 minutes, respectively. A standard curve was
prepared using the 21-mer peptide to allow quantitation
35 of product formation. Effect of incubation time was
evaluated and found to be linear over the timecourse of
the assay (Figure 2).

5

Example 3

Inhibition of ADMP Activity by a Peptide Inhibitor

A peptide inhibitor was prepared based upon the sequence of the 40 amino acid peptide SEQ ID NO:1, but designed such that it contained a Glu to Gln substitution at the P1 of the Glu373-Ala374 bond. This peptide (30-IP) of the sequence:

QTVTWPDMELPLPRNITEGQARGSVILTVK-Biotin

(SEQ ID NO:7)

was prepared with the n-terminus acetylated and the c-terminal residue present as the amide (Quality Controlled Biochemicals, Inc., Hopkinton, MA). This peptide was employed in a microplate assay as described in Example 1 wherein the 30-IP inhibitor was substituted for the hydroxamate inhibitor used in that example. The 30-IP inhibitor was employed at final concentrations of 0.01, 0.1, 1.0, 3.0, 5.0, 10.0, 30.0 and 100.0 μ M.

ADMP activity was inhibited as shown in Figure 3. The IC_{50} for the inhibition of ADMP activity by 30-IP was 11 μ M.

EXAMPLE 4

Problot Assay

This example describes a method for analyzing ADMP enzymatic activity and inhibitors of this activity by monitoring cleavage at the E373-A374 bond using the BC-3 antibody to detect fragments with the new N-terminus, ARGS. Samples containing ADMP activity (10 units/ml) were incubated with 500 nM bovine aggrecan monomer in a final volume of 200 μ L in 0.05 M Tris, pH 7.6, containing 0.1 M NaCl and 10 mM $CaCl_2$. Reactions were incubated for 4 hr at 37°C, quenched with 20 mM EDTA, and analyzed for aggrecan fragments with the new N-terminus, ARGS,

5 generated by specific ADMP-mediated cleavage using the
Problot assay.

10 The Immobolin PVDF membrane plate (#MAIPN4550;
Millipore Corp., Bedford, MA) was prewet with 50 μ L per
well 70% ethanol, incubated for 30 seconds at room
temperature then flushed two times each with 200 μ L of
purified H₂O. The plate was then coated with aggrecan
equivalent to 36 μ g of glycosaminoglycan (GAG) as
detected by the dimethyl methylene blue dye assay
[Farndale R.W., et al., (1982) Conn. Tiss. Res. 9, 247-
248.] in 150 μ L of 50 mM carbonate-bicarbonate buffer, pH
9.6, overnight at 4°C with gentle agitation. The coating
solution was then filtered off of the plate using a
vacuum manifold and membranes were washed once with 200
 μ L of Buffer A (Buffer A comprises 20 mM Tris, 500 mM
NaCl, pH 7.5) allowing 20 seconds of contact with the
membrane. Membranes were then blocked with 150 μ L of 5%
BSA/TBS solution for 1 hour at room temperature with
gentle agitation. The blocking solution was filtered off
of the plate and the membranes washed one time with 200
 μ L of 1X TBS buffer, allowing 20 seconds of contact with
membrane per wash.

30 Removal of the glycosaminoglycan (GAG) side chains
from aggrecan is necessary for the BC-3 antibody to
recognize the epitope on the core protein. Therefore, to
remove GAGs from the bound aggrecan, samples were treated
with deglycosylation enzymes as follows: 0.01 units
chondroitinase ABC (#EC4.2.2.4; Seikagaku Co., Kogyo,
Japan) per 10 μ g GAG in 150 μ L of Buffer B (Buffer B
comprises 50 mM sodium acetate, 100 mM NaCl, pH 6.5) was
added to each well and incubated at 37°C for 1 hour.
Following incubation the enzyme solution was filtered out
of the plate and 0.01 units chondroitinase ABC per 10 μ g
GAG, 0.01 units keratanase I (#EC3.2.1.103; Co., Kogyo,
Japan) per 10 μ g GAG, and 0.0005 units keratanase II
(Seikagaku Co., Kogyo, Japan) per 10 μ g GAG in 150 μ L
Buffer B were added and allowed to incubate an additional

5 2 hours at 37°C. Enzyme solution was filtered out and membranes rinsed one time with 200 µL of Buffer A.

150 µL of BC-3 antibody was added at a 1:500 dilution in 1% BSA in Buffer A and incubated for 1 hour at room temperature with gentle agitation. BC-3 antibody
10 was removed and membranes washed three times each with 200 µL Buffer A allowing membrane contact for 20 seconds per wash. Next 150 µL of goat anti-mouse IgG AP conjugate (#S3721; Promega, Madison, WI) was added at a 1:2500 dilution in 1% BSA/TBS buffer and allowed to
15 incubate for 1 hour at room temperature with gentle agitation. The secondary antibody was prefiltered prior to use, using 0.22 µm syringe filters to remove aggregates which cause high background. Following the incubation, secondary antibody was removed and wells were
20 washed three times each with 200 µL Buffer A allowing contact for 20 seconds per wash. Then 100 µL of p-NPP AP substrate solution (# 50-80-00; Kirkegaard & Perry Lab., Gaithersburg, MD) was added to each well and incubated in the dark at room temperature for 30 minutes. The
25 solution was then filtered into a corresponding ELISA plate. The filter plate was washed with 100 µL of 500 mM EDTA and the wash was combined with the corresponding samples in the ELISA plate. Absorbance of the samples was read at 405 nm (Thermomax plate reader).

30 By coating the plate with the target peptide, ARGS, linked to BSA representing the ADMP-generated product, one can create a standard curve which can be used to convert optical density values to units of activity. A unit of ADMP activity is defined as that resulting in
35 product produced equivalent to 1 µg BSA peptide per hour at 37°C.

To evaluate inhibition of ADMP activity, compounds are prepared as 10 mM stocks in dimethyl sulfoxide (DMSO), water or other solvents and diluted to
40 appropriate concentrations in water. Drug (50 µL) was added to 50 µL of 2 mg/mL aggrecan substrate and 50 µL of ADMP (40 units/ml) and brought to a final volume of 200

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5 μ L by addition of 50 μ l of 0.2 M Tris, pH 7.6,
containing 0.4 M NaCl and 40 mM CaCl_2 . The reaction
mixture was incubated for 4 hr at 37°C, quenched with 20
mM EDTA and analyzed for ADMP-generated products. A
sample containing enzyme and substrate without drug was
10 included as a positive control and enzyme prequenched
with EDTA served as a measure of background.

IC₅₀ values for inhibitors of ADMP enzymatic
activity determined using the Problot assay with the BC-3
antibody for analysis of product generation by cleavage
15 at the E373-A374 bond correlated ($r^2=0.99$) with those
determined using a BC-3 Western blot analysis to detect
product formation.

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